

Protective effect of *Carica papaya* leaves against oxidative stress in brine shrimps

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ABSTRACT

Carica papaya, a type of tree from the *Caricaceae* family, is renowned for its medicinal benefits due to its diverse chemical composition. The leaves of *Carica papaya*, in particular, are commonly used in traditional medicine for their purported anti-dengue, anti-malarial, antioxidant, and anti-inflammatory properties. However, despite their widespread use, there is limited research available on the antioxidant characteristics of *Carica papaya* leaves. This study aimed to examine the potential protective effects of *Carica papaya* leaves methanolic (CPLM) extract against oxidative stress in *Artemia salina* (brine shrimp). To evaluate the antioxidant potential of CPLM extract, two commonly used antioxidant activity assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) assays, were performed. Additionally, an oxidative stress protection assay was carried out by exposing ten *Artemia salina* nauplii to CPLM extract for one hour before exposing them to hydrogen peroxide (H_2O_2). The results of this study showed that the CPLM extract has antioxidant properties. Additionally, the oxidative stress protection assay revealed that the CPLM extract increased the survival rate of *Artemia salina* nauplii exposed to H_2O_2 . The study suggests that the CPLM extract obtained from *Carica papaya* leaves possesses antioxidant properties. It can provide protection against oxidative stress caused by hydrogen peroxide in *Artemia salina*.

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1. INTRODUCTION

Carica papaya, commonly referred to as papaya, is a tree species classified under the family Caricaceae. Almost every component of the plant (fruit, leaf, flower, seeds, latex, and roots) has unique medicinal benefits [1]. Over recent decades, numerous studies have been conducted to verify the medicinal and pharmacological uses of papaya trees such as anti-diabetic, anti-malarial, anti-dengue, and also antioxidant [2]–[6]. The therapeutic properties of papaya trees are derived from different parts of the plant, including the leaves, fruits, seeds, and roots. Each part contains specific phytoconstituents that have potential medicinal benefits [7].

This study focuses on the leaves part of *Carica papaya* as it is considered the greatest component of *Carica papaya* since possessing many medicinal values [8]. The leaves of *Carica papaya* contain numerous phytochemicals such as steroids, saponins, tannins, alkaloids, flavonoids, and vitamins [9]–[11]. Minerals like

calcium, magnesium, potassium, and iron are also found in the leaves of *Carica papaya* [12], [13]. Although several studies have reported that *Carica papaya* possesses anti-dengue [14], anti-inflammatory [15], antioxidant effects [16], nephroprotective agents [17], and anti-diabetic properties [18], most of these impressive presumptive benefits are linked to the anti-inflammatory and antioxidant characteristics of the *Carica papaya* leaves. The high-performance liquid chromatography (HPLC) quantitative analysis of *Carica papaya* leaves revealed a significant amount of flavonoids, specifically kaempferol 3-(2G-rhamnosylrutinoside), that exhibit potent antioxidant activities compared to other identified compounds [16]. Subsequently, they have potent antioxidant effects and can help neutralize free radicals, such as reactive oxygen species (ROS), due to their inherent redox properties [14], [16], [19]. ROS, such as superoxide anion and hydrogen peroxide, are reactive oxygen intermediates normally generated in the cell (mainly the mitochondria); however, when antioxidants and free radicals are imbalanced, it can lead to severe oxidative stress [20].

As an alternative for medicinal plant toxicological evaluation, this study uses brine shrimps, which are invertebrate organisms scientifically known as *Artemia salina*. The affordability, repeatability, robustness, simplicity, and rapidity are the advantages of using brine shrimps [21]. Previous toxicological evaluations of the *Carica papaya* extracts conducted using brine shrimps mostly used ethanol and aqueous as solvents in the extraction process [22], [23]. However, there are only limited numbers of toxicity studies of *Carica papaya* leaves methanolic (CPLM) extract. The CPLM extract contains more phytochemicals and exhibits higher antioxidant activities in comparison to other solvents such as ethanol [9]. However, despite its apparent usage and advantages for health, there is insufficient information regarding the toxic doses of CPLM extract in brine shrimp and the safe dose that is capable of inducing the antioxidative activity. Therefore, the aim of this study was to determine a safe dose for the CPLM extract and to evaluate its efficacy in protecting brine shrimp against oxidative stress. This study is a significant endeavor in ensuring the safe consumption of CPLM extract and proving the protective effect of CPLM extract against oxidative stress.

2. METHOD

2.1. Extract preparation

The *Carica papaya* leaves were gathered from the Faculty of Pharmacy, UiTM Selangor, Puncak Alam Branch, Malaysia and processed to produce the CPLM extract via maceration technique. First, the leaves were cleansed and dehydrated at 40 °C, then pulverized into a fine powder. The powder was blended with methanol at a proportion of 1:10 and allowed to macerate for three days with occasional stirring. Next, the mixture was filtered through Whatman filter paper No.1, and the maceration process was performed thrice. After filtration, the obtained filtrate was subjected to drying using a rotary evaporator at a temperature of 40-50 °C for a duration of 8-10 minutes. The dried residue was then transferred into a container and kept in an oven at a temperature of 40 °C for three days. The resultant extract was stored in a cool place at a temperature of 4 °C until it was ready to be used.

2.2. Phytochemical screening

To confirm the presence of saponins in the CPLM extract, a 100 mg sample of the extract was taken and mixed with distilled water in a test tube. The presence of saponins can be detected by observing the formation of a persistent stable foam that lasts for at least 15 minutes. This foam formation is a result of the ability of saponins to lower surface tension, which leads to the formation of stable bubbles in the solution. The formation of stable foam is thus considered a characteristic feature of saponins and is used as an indicator of their presence in the extract.

To investigate the presence of tannins and polyphenolic compounds in the CPLM extract, a 100 mg sample was taken and mixed with a 1% solution of ferric chloride. When hydrolysable tannins are present, the solution will show a blue-black color. On the other hand, if condensed tannins are present, the solution will show a brownish-green color.

To determine the presence of alkaloids in the CPLM extract, a 100 mg sample was first mixed with chloroform. Then, the chloroform extract was further treated with ammoniacal chloroform and 10% sulfuric acid. This was done in order to remove any impurities and concentrate the alkaloids present in the extract. To confirm the presence of alkaloids, Mayer's reagent was used to test the resulting mixture. If the solution turned cloudy or produced a yellowish precipitate, it was an indication of the presence of alkaloids in the CPLM extract.

To detect flavonoids in the CPLM extract, a 100 mg sample was mixed with 5 mL of 95% ethanol and heated in a water bath. The solution was then filtered, and a few drops of 1% aluminum chloride were added. The formation of a yellow color indicated the presence of flavonoids.

To detect triterpenes and steroids in the CPLM extract, the chloroform mixture was evaporated to dryness, and the residue was dissolved in 1 mL of acetic anhydride. A few drops of concentrated sulfuric acid

were added, and the resulting mixture observed a color change. The formation of a violet color indicated the presence of triterpenes, while the formation of a blue-green color indicated the presence of steroids.

2.3. Antioxidant activity assays

The ability of the CPLM extract to scavenge free radicals was assessed using two widely used antioxidant assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the Ferric reducing antioxidant power (FRAP) assay. The DPPH assay involves the reduction of the stable DPPH radical by an antioxidant compound resulting in a color change that can be measured spectrophotometrically. The FRAP assay measures the ability of a compound to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in a redox reaction, which can be measured by the formation of a blue color. The procedures for conducting the DPPH and FRAP assays were performed as described in previous studies [24], [25].

2.4. Brine shrimp lethality test

To create a saltwater solution, 30 g of instant ocean salt was dissolved in one liter of distilled water. Then, 0.15 g of *Artemia salina* cysts were placed in 250 ml of the saltwater solution and kept at 28 °C with sufficient lighting and aeration. After 24 hours of incubation, the nauplii were collected.

The lethality tests on brine shrimp were conducted using a previously described method with some modifications [26], [27]. The procedure involved preparing five different concentrations of CPLM extract (0.5, 1, 2, 4, and 6 mg/ml) in triplicates through serial dilution with 1% dimethyl sulfoxide (DMSO) in salt water. In each well of a 24 well-plate, 10 nauplii were placed with 1 ml of the CPLM extract. Negative control wells contained ten nauplii and 1 ml of 1% DMSO in salt water, while positive control wells contained ten nauplii and 1 ml of 0.2 mg/ml potassium dichromate.

After adding the CPLM extract to the nauplii, the 24-well plate was kept at a temperature of 28 °C for 24 hours. The mortality percentage was determined by counting the dead nauplii after incubation. The trial was conducted in triplicate to ensure the precision and reliability of the outcomes.

2.5. Oxidative stress protection assay

The nauplii were obtained using a method similar to the brine shrimp lethality test, and five safe concentrations of the CPLM extract were selected based on the brine shrimp lethality test results. The CPLM extract was dissolved in salt water with 1% DMSO, and ten nauplii were exposed to 1 ml of the solution in each well. The control group was treated with 1 ml of salt water. The well plate was then incubated at 28°C for one hour.

Following the 1-hour exposure to CPLM extract, the nauplii were subjected to a solution of hydrogen peroxide (H_2O_2) diluted in salt water for 24 hours, with the concentration of H_2O_2 being selected based on the LC_{50} value in brine shrimps. The well plate was then incubated at 28 °C, and the survival of the nauplii was observed under a microscope after the 24-hour incubation period. The experimental procedure was performed in triplicate.

2.6. Statistical analysis

To determine statistical differences between means, one-way ANOVA and t-tests were utilized and analyzed with GraphPad Prism 9. The level of significance chosen for the study was 0.05, meaning that any p-value less than 0.05 was considered to be statistically significant. Probit regression analysis was also conducted with Microsoft Excel, and Finney's table was applied to convert percentage mortality to probit value. The LC_{50} values were obtained by regression analysis of the best-fit line, which signifies the concentration required to cause 50% mortality in the sample population of the extract [21].

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

The results in Table 1 indicate that the CPLM extract contained saponins, condensed tannins, and flavonoids, as detected in the phytochemical screening. However, it is worth noting that a previous study conducted in India reported the presence of alkaloids but not tannins in the CPLM extract [28]. This discrepancy may be due to the geographical origin of the *Carica papaya* plant which may affect the phytochemical constituents of the extract. The occurrence of saponins, flavonoids, and tannins in the CPLM extract could suggest that the extract possesses antioxidant properties. Flavonoids, which are a category of naturally occurring molecules, are recognized for their therapeutic advantages and antioxidant effects [29]. A double bond, 4-carbonyl groups, hydroxyl groups, and O-methylation in flavonoids are essential since these chemical groups are involved in flavonoids' antioxidant potential [29]. Additionally, flavonoids in the leaves can inhibit the activities of phenolic hydroxyl groups; as a consequence, flavonoids can transfer hydrogen

and facilitate the delocalization of phenoxy radical products, preventing various diseases caused by a buildup of reactive oxygen species (ROS) [30].

Table 1. Phytochemical screening of *Carica papaya* leaves methanolic extract

Test	Result
Saponins	Detected
Tannins and polyphenolic compounds	Condensed tannins were detected
Alkaloids	Not detected
Flavonoids	Detected
Triterpenes	Not detected
Steroids	Not detected

3.2. Antioxidant activity of CPLM extract

The DPPH assay was used to evaluate the antioxidant activity of the CPLM extract, which determines the extract's potential to function as either a hydrogen donor or a scavenger of free radicals. The results of the assay are presented in Figure 1, with Figure 1(a) showing the average percentage of DPPH inhibition for the CPLM extract and Figure 1(b) showing the average percentage of DPPH inhibition for ascorbic acid. According to the results, the IC_{50} values of the CPLM extract and ascorbic acid were 1.11 mg/ml and 8.73 μ g/ml, respectively. Ascorbic acid is a powerful antioxidant, and its IC_{50} value was found to be lower than that of the CPLM extract. A lower IC_{50} value indicates stronger free radical scavenging activity and a more potent extract as an antioxidant [19], [31]. Therefore, the findings suggest that the CPLM extract is not as effective as ascorbic acid in terms of its antioxidant properties. Previous research has demonstrated that among different solvents used to extract *Carica papaya* leaves, the CPLM extract exhibited the strongest capacity to eliminate DPPH free radicals, with water and 70% ethanol following closely behind [32]. Moreover, the study observed a positive correlation between the antioxidant activity of *Carica papaya* leaves extracts and the total flavonoid concentration in the sample. Therefore, the current study's findings on the strong antioxidant potential of the CPLM extract align with the previous research and suggest that the extract's high flavonoid content could be responsible for its radical scavenging abilities.

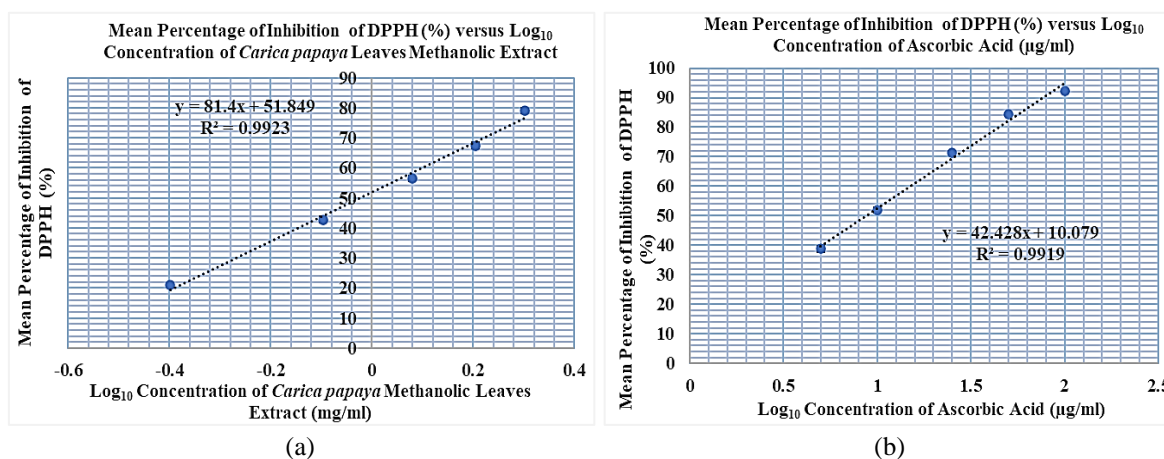


Figure 1. Graph of mean percentage of inhibition of DPPH (%) versus log10 concentration of (a) CPLM extract (mg/ml) and (b) ascorbic acid (μ g/ml)

The study evaluated the ability of CPLM extracts to neutralize ferric ions using FRAP assays. Figure 2 displays that the FRAP values had a linear relationship with the CPLM extract concentration. The extract showed higher reduction capacity compared to ascorbic and gallic acids, as illustrated in Figures 2(a) and 2(b), respectively [33]. The relationship between the concentration of CPLM leaves and FRAP values observed in this study suggests that the extract possesses a high potential to reduce ferric ions, making it an effective antioxidant agent. This could be attributed to the presence of flavonoids and phenols in the extract, as reported in previous studies [32], [34]. The flavonoids and phenols present in the extract are known to possess strong antioxidant properties, which can help in reducing oxidative stress and preventing damage to

cellular structures. Therefore, the results of this study suggest that the CPLM extract could be used as a potential source of natural antioxidants.

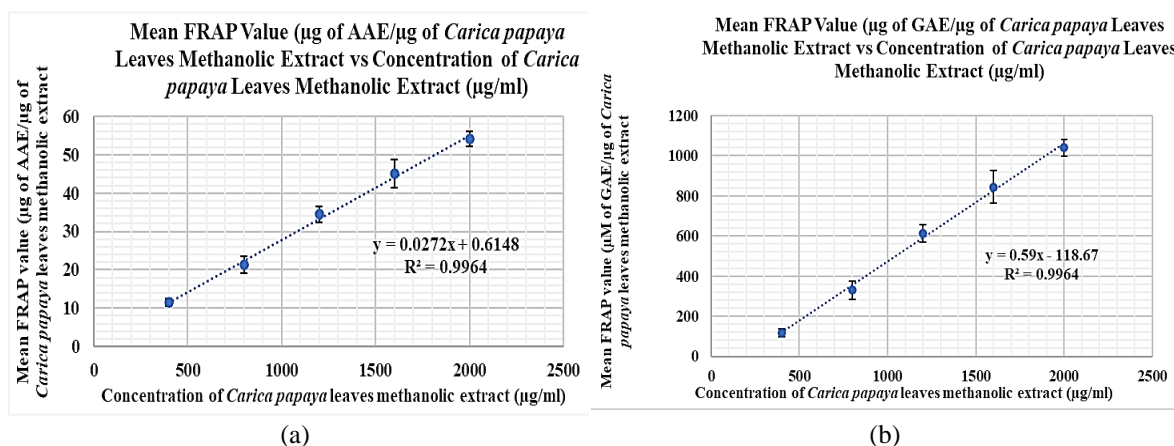


Figure 2. Graph of mean of FRAP value (a) (µg of AAE/µg of CPLM extract) vs concentration of CPLM extract (µg/ml) and (b) (µM of GAE/µg of CPLM extract) vs concentration of CPLM extract (µg/ml)

3.3. Oxidative stress protection of CPLM extract in brine shrimps

Figure 3 illustrates the ability of CPLM extract to protect brine shrimps against oxidative stress. Figure 3(a) depicts the mortality percentage of *Artemia salina* after being exposed for 24 hours to different solutions and concentrations of CPLM extract. The LC₅₀ value obtained from the probit regression analysis was 2.07 mg/ml for the CPLM extract. The toxicity of a plant extract can be evaluated based on its LC₅₀ value using the Clarkson index. A highly toxic extract has an LC₅₀ value of 0-100 µg/ml, a moderately toxic extract has an LC₅₀ value of 100-500 µg/ml, while a slightly toxic extract has an LC₅₀ value of 500-1,000 µg/ml [21]. Extracts with LC₅₀ values above 1,000 µg/ml are considered non-toxic [21]. Based on this index, the CPLM extract can be considered non-toxic since its LC₅₀ value exceeded 1,000 µg/ml. These results suggest that the CPLM extract is safe for consumption and has the potential as a natural antioxidant.

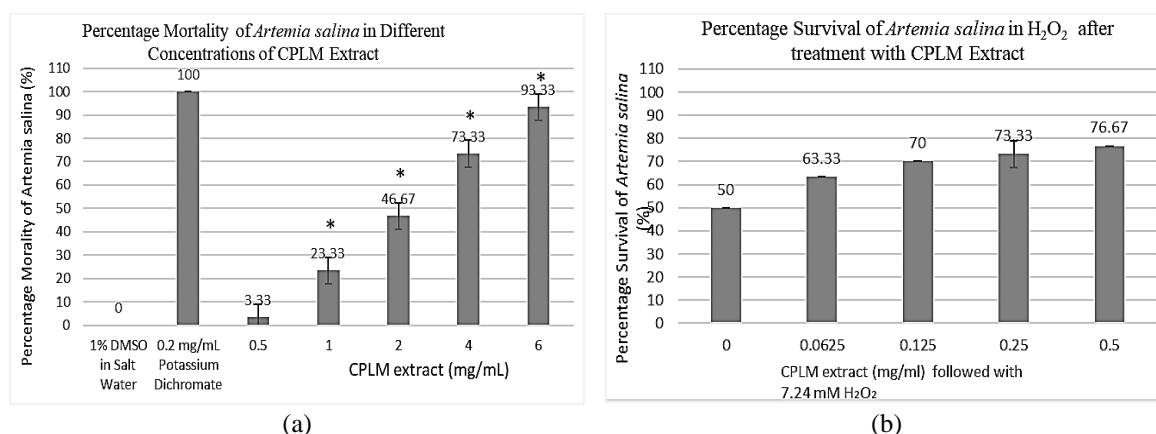


Figure 3. Percentage of (a) mortality of *Artemia salina* after 24 hours being exposed to 1% DMSO in salt water, 0.2 mg/ml potassium dichromate and different concentrations CPLM extract (*indicates statistically significantly difference ($p < 0.05$) compared to 1% DMSO in salt water) and (b) survival of *Artemia salina* after 24 hours being treated with CPLM extract and exposed to H₂O₂

Figure 3(b) displays the survival rate of *Artemia salina* following treatment with CPLM extract for one hour, and subsequent exposure to 7.24 mM H₂O₂ for 24 hours. The survival of *Artemia salina* was 50% when they were not treated with CPLM extract prior to the exposure to H₂O₂. However, the survival of

Artemia salina increased when being treated with CPLM extract. This indicated that the CPLM extract could provide a protective effect to *Artemia salina* against oxidative stress induced by H₂O₂.

Hydrogen peroxide has been used as an oxidative stress inducer in this study. Hydrogen peroxide is a non-radical reactive oxygen species but capable of generating hydroxyl radicals that are highly reactive which eventually cause damage to cells [35]. This study found that higher concentrations of CPLM extract resulted in a greater percentage of survival for *Artemia salina*, suggesting that the extract's ability to provide protection against oxidative stress induced by H₂O₂ may be influenced by its concentration. There are few studies that have demonstrated an increase in free radical scavenging activity of *Carica papaya* leaves extract as the concentration of the extract was increased [6], [9], [36]. The analysis of the *Carica papaya* leaves extract revealed a high concentration of both flavonoid and phenolic compounds [37]. Additionally, *Carica papaya* leaves also contain vitamins such as Vitamin C, E, and beta-carotene [38]. Vitamin C is a well-known antioxidant that exhibits its antioxidant activity through a few mechanisms. For instance, by serving as a reducing agent since it acts as a hydrogen atom donor to free radical compounds and yields an ascorbyl-free radical stable molecule [39]. The leaves of *Carica papaya* contain enzymes like papain which can induce antioxidant activity by donating hydrogen atoms from the amino acids in its chemical structure [40]. This, along with the presence of other antioxidants such as phenols, flavonoids, vitamins, papain, and chymopapain in the leaves, can contribute to the overall antioxidant effect of the leaves [37], [38], [40].

These findings suggest that the CPLM extract may have the potential as a natural antioxidant and could be used in the development of functional foods and nutraceuticals. Further studies are warranted to investigate the mechanisms behind the protective effects of the CPLM extract and to evaluate its efficacy in other in vivo and in vitro models. Nevertheless, the results of this study support the traditional use of *Carica papaya* leaves as a medicinal plant with potential health benefits.

4. CONCLUSION

In conclusion, the CPLM extract was shown to exhibit antioxidant activity. In addition, the extract was shown to provide protective effects to *Artemia salina* against oxidative stress induced by hydrogen peroxide. These findings highlight the potential health benefits of *Carica papaya* leaves and their potential as a natural antioxidant source. Further studies are warranted to explore the potential of CPLM extract as a natural antioxidant agent and its potential applications in human health.

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
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